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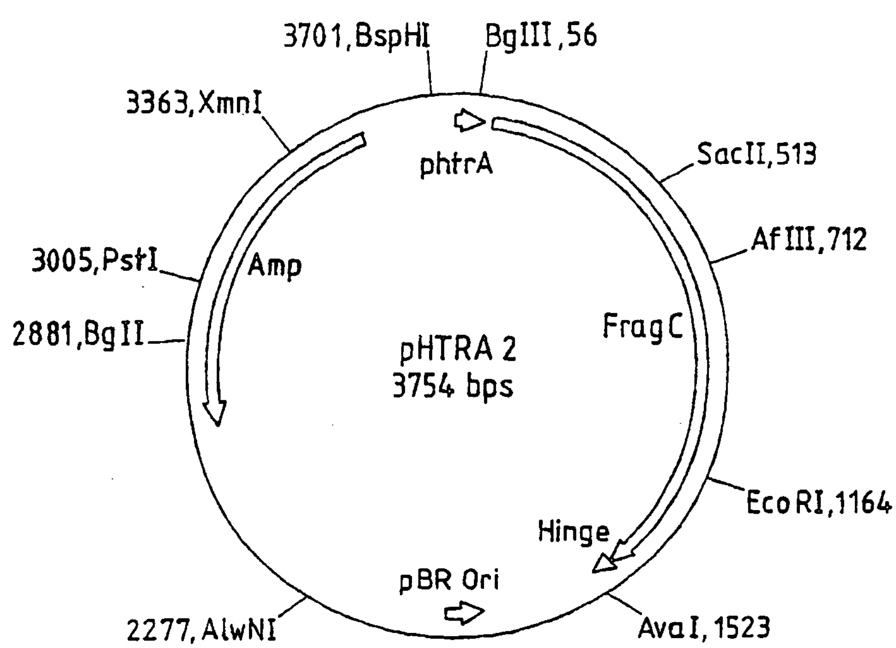

(57) Abstract

The invention provides a DNA construct comprising the htrA promoter sequence operably linked to a DNA sequence encoding one or more heterologous proteins, replicable expression vectors containing the constructs, and attenuated bacteria containing the constructs or vectors. The invention also provides a vaccine composition comprising an attenuated bacterium as defined above, or a fusion protein expressed from a construct as defined above, and a pharmaceutically acceptable carrier.

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VACCINES

EXPRESSION OF HETEROLOGOUS PROTEINS IN ATTENUATED BACTERIA USING THE HTRA-PROMOTERS.

This invention relates to DNA constructs, replicable expression vectors containing the constructs, attenuated bacteria containing the constructs and vaccines containing the said bacteria.

In recent years, there has emerged a new generation of live oral salmonella vaccines based upon strains of Salmonella which have been attenuated by the introduction of a non-reverting mutation in a gene in the aromatic biosynthetic pathway of the bacterium. Such strains are disclosed, for example, in EP-A-0322237. The aforesaid live oral salmonella vaccines are showing promise as vaccines for salmonellosis in man and animals, and they can also be used effectively as carriers for the delivery of heterologous antigens to the immune system. Combined salmonella vaccines have been used to deliver antigens from viruses, bacteria, and parasites, eliciting secretory, humoral and cell-mediated immune responses to the recombinant antigens. Combined salmonella vaccines show great potential as single dose oral

multivaccine delivery systems [C. Hormaeche et al, FEMS Symposium No. 63, Plenum,New York; pp 71-83, 1992].

There are problems to be overcome in the development of combined salmonella vaccines. A major consideration is obtaining a high level of expression of the recombinant antigen in the salmonella vaccine so that it will be sufficient to trigger an immune response. However, unregulated high level expression of foreign antigens can be toxic and affect cell viability [I. Charles and G. Dougan, TIBTECH 8, pp 117-21, 1990], rendering the vaccine ineffective or causing loss of the recombinant DNA. Several possible solutions to this problem have been described, such as expression from plasmids carrying essential genes, "on-off" promoters or incorporation of the foreign genes into the salmonella chromosome.

An alternative approach to overcoming the aforesaid problem would be to use a promoter which is inducible in vivo, and one such promoter is the E.coli nitrite reductase promoter nirB which is induced under anaerobiosis. Vaccine compositions containing bacteria transformed with constructs comprising the nirB promoter are described in our earlier International Patent Application PCT/GB93/01617.

The present invention relates to the preparation of DNA constructs containing a different inducible promoter, namely the promoter for the htrA gene which encodes a stress induced protein.

The htrA gene is described in K. Johnson et al Mol. Microbiol 1991; 5:401-7 and references cited therein and is an example of a gene encoding a heatshock protein which is produced in response to a temperature increase above 42°C.

Accordingly, in a first aspect, the invention provides a DNA construct comprising the htrA promoter sequence operably linked to a DNA sequence encoding one or more heterologous proteins.

In one embodiment, the invention provides a DNA construct as hereinbefore defined wherein the htrA promoter sequence is operably linked to a DNA sequence encoding a fusion protein of two or more heterologous proteins.

The proteins making up the fusion may be linked by means of a flexible hinge region.

In a further aspect, the invention provides a DNA construct comprising the htrA promoter sequence operably linked to a DNA sequence encoding first and second heterologous proteins wherein the first heterologous protein is an antigenic sequence comprising tetanus toxin fragment C or one or more epitopes thereof.

In a further aspect, the invention provides a replicable expression factor, suitable for use in bacteria, containing a DNA construct as hereinbefore defined.

In another aspect, the invention provides a fusion protein, preferably in substantially pure form, the fusion protein being expressed by a construct as hereinbefore

defined.

In a further aspect, the invention provides a process for the preparation of an attenuated bacterium which comprises transforming an attenuated bacterium with a DNA construct as hereinbefore defined.

In a still further aspect, the invention provides a host cell, such as a bacterial cell, containing a DNA construct as hereinbefore defined. The DNA construct may be present in extra-chromosomal form, e.g. in a plasmid, or may be integrated into the host (e.g. bacterial) chromosome by methods known <u>per se</u>.

The invention also provides a vaccine composition comprising an attenuated bacterium as hereinbefore defined, or a fusion protein expressed therefrom, and a pharmaceutically acceptable carrier.

The first and second proteins are preferably heterologous proteins and in particular can be polypeptide immunogens; for example they may be antigenic sequences derived from a virus, bacterium, fungus, yeast or parasite. In particular, it is preferred that the first said protein is an antigenic sequence comprising tetanus toxin fragment C or epitopes thereof.

The second protein is preferably an antigenic determinant of a pathogenic organism. For example, the antigenic determinant may be an antigenic sequence derived from a virus, bacterium, fungus, yeast or parasite.

Examples of viral antigenic sequences for the first and/or second heterologous proteins are sequences derived from a type of human immunodeficiency virus (HIV) such as HIV-1 or HIV-2, the CD4 receptor binding site from HIV, for example from HIV-1 or -2, hepatitis A, B or C virus, human rhinovirus such as type 2 or type 14, Herpes simplex virus, poliovirus type 2 or 3, foot-and-mouth disease virus (FMDV), rabies virus, rotavirus, influenza virus, coxsackie virus, human papilloma virus (HPV), for example the type 16 papilloma virus, the E7 protein thereof, and fragments containing the E7 protein or its epitopes; and simian immunodeficiency virus (SIV).

Examples of antigens derived from bacteria are those derived from Bordetella pertussis (e.g. P69 protein and filamentous haemagglutinin (FHA) antigens), Vibrio cholerae, Bacillus anthracis, and E.coli antigens such as E.coli heat Labile toxin B subunit (LT-B), E.coli K88 antigens, and enterotoxigenic E.coli antigens. Other examples of antigens include the cell surface antigen CD4, Schistosoma mansoni P28 glutathione S-transferase antigens (P28 antigens) and antigens of flukes, mycoplasma, roundworms, tapeworms, Chlamydia trachomatis, and malaria parasites, eg. parasites of the genus plasmodium or babesia, for example Plasmodium falciparum, and peptides encoding immunogenic epitopes from the aforementioned antigens.

Particular antigens include the full length Schistosoma

mansoni P28, and oligomers (e.g. 2, 4 and 8mers) of the immunogenic P28 aa 115-131 peptide (which contains both a B and T cell epitope), and human papilloma virus E7 protein, Herpes simplex antigens, foot and mouth disease virus antigens, simian immunodeficiency virus antigens, and the diphtheria toxin antigens, e.g. the diphtheria toxin ganglioside binding region.

As used herein, references to the htrA promoter refer to the promoter itself or a part or derivative thereof which is capable of promoting expression of a coding sequence. The preferred sequence, and which contains the htrA promoter is:
AATTCTATTCCGGAACTTCGCGTTATAAAATGAATCTGACGTACACAGCAATTTA
(SEQ.ID.NO.1)

In the constructs of the present invention, the DNA sequence may encode a fusion protein of two or more proteins in which adjacent proteins are separated by a hinge region. The hinge region is a region designed to promote the independent folding of both the first and second proteins by providing both spatial and temporal separation between the domains.

The hinge region typically is a sequence encoding a high proportion of proline and/or glycine amino acids. The hinge region may be composed entirely of proline and/or glycine amino acids. The hinge region may comprise one or more glycine-proline dipeptide units.

The hinge region may, for example, contain up to about

fifteen amino acids, for example at least 4 and preferably 6-14 amino acids, the number of amino acids being such as to impart flexibility between the first and second proteins.

In one embodiment, the hinge region can correspond substantially to the hinge domain of an antibody immunoglobulin. The hinge regions of IgG antibodies in particular are rich in prolines [T.E. Michaelson et al. J. Biol. Chem. 252, 883-9 1977], which are thought to provide a flexible joint between the antigen binding and tail domains.

Without wishing to be bound by any theory, the prolines are thought to form the rigid part of the hinge as the ring structure characteristic of this amino acid hinders rotation around the peptide bond that connects the proline residue with an adjacent amino acid. This property is thought to prevent proline, and adjacent residues, from adopting the ordered structure of an alpha helix or beta strand. Flexibility is thought to be imparted by glycine, the simplest amino acid, with very limited steric demands. Glycine is thought to function as a flexible elbow in the hinge. Other amino acids may be substituted for glycine, particularly those without bulky side-chains, such as alanine, serine, asparagine and threonine.

In one preferred embodiment, the hinge region is a chain of four or more amino acids defining the sequence

$$-[X]_p\text{-Pro-}[Y]_q\text{-Pro-}[Z]_r-$$

wherein Pro is proline, X and Y are each glycine, or an amino

acid having a non-bulky side chain; Z is any amino acid; p is a positive integer; q is a positive integer of from one to ten; and r is zero or a positive integer greater than zero.

The hinge region can be a discrete region heterologous to both the first and second proteins or can be defined by a carboxy-end portion of the first protein or an amino-end portion of the second protein.

In a most preferred aspect, the present invention provides a DNA molecule comprising the htrA promoter operably linked to a DNA sequence encoding first and second polypeptide immunogens linked by a hinge region, wherein the first polypeptide immunogen comprises tetanus toxin fragment C or epitopes thereof.

In another preferred aspect of the invention, there is provided a replicable expression vector, suitable for use in bacteria, containing the htrA promoter sequence operably linked to a DNA sequence encoding first and second polypeptide immunogens linked by a hinge region, wherein the first polypeptide immunogen comprises tetanus toxin fragment C or epitopes thereof.

In a further aspect, the invention provides a DNA construct comprising the htrA promoter operably linked to DNA encoding a first protein and, extending from the 3' end thereof a DNA sequence encoding the hinge region, and downstream thereof, one or more restriction endonuclease sites.

The said protein is preferably an antigenic protein as hereinbefore defined, and in particular is the TetC fragment or epitopes thereof.

Stable expression of the first and second heterologous proteins linked by the hinge region can be obtained *in vivo*. The heterologous proteins can be expressed in an attenuated bacterium which can thus be used as a vaccine.

The attenuated bacterium may be selected from the genera *Salmonella*, *Bordetella*, *Vibrio*, *Haemophilus*, *Neisseria* and *Yersinia*. Alternatively, the attenuated bacterium may be an attenuated strain of enterotoxigenic *Escherichia coli*. In particular the following species can be mentioned: *S.typhi* - the cause of human typhoid; *S.typhimurium* - the cause of salmonellosis in several animal species; *S.enteritidis* - a cause of food poisoning in humans; *S.choleraesuis* - a cause of salmonellosis in pigs; *Bordetella pertussis* - the cause of whooping cough; *Haemophilus influenzae* a cause of meningitis; *Neisseria gonorrhoeae* - the cause of gonorrhoea; and *Yersinia* - a cause of food poisoning.

Attenuation of the bacterium may be attributable to a non-reverting mutation in a gene in the aromatic amino acid biosynthetic pathway of the bacterium. There are at least ten genes involved in the synthesis of chorismate, the branch point compound in the aromatic amino acid biosynthetic pathway. Several of these map at widely differing locations on the bacterial genome, for example *aroA* (5-

enolpyruvylshikimate-3-phosphate synthase), aroC (chorismate synthase), aroD (3-dihydroquinate dehydratase) and aroE (shikimate dehydrogenase). A mutation may therefore occur in the aroA, aroC, aroD, or aroE gene.

Preferably, however, an attenuated bacterium harbours a non-reverting mutation in each of two discrete genes in its aromatic amino acid biosynthetic pathway; or harbours a non-reverting mutation in its aromatic biosynthetic pathway and a non-reverting mutation in a regulatory gene such as htrA, OmpR or OsmC. Examples of suitable attenuated bacteria are disclosed in, for example, EP-A-0322237, and EP-A-0400958.

An attenuated bacterium containing a DNA construct according to the invention can be used as a vaccine. Fusion proteins (preferably in substantially pure form) expressed by the bacteria can also be used in the preparation of vaccines. For example, a purified TetC-P28 fusion protein has been found to be immunogenic on its own. In a further aspect therefore, the invention provides a vaccine composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, an attenuated bacterium or fusion protein as hereinbefore defined.

The vaccine may comprise one or more suitable adjuvants.

The vaccine is advantageously presented in a lyophilised form, for example in a capsular form, for oral administration to a patient. Such capsules may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L",

Cellulose acetate, Cellulose acetate phthalate or Hydroxypropylmethyl Cellulose. These capsules may be used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is advantageously effected in buffer at a suitable pH to ensure the viability of the organisms. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively, the vaccine may be prepared for parenteral administration, intranasal administration or intramammary administration.

Preferably, the vaccine composition is adapted for mucosal delivery, eg by oral administration, by intranasal administration or by intrabronchial administration.

The attenuated bacterium containing the DNA construct of the invention may be used in prophylaxis or treatment of a host, particularly a human host but also possibly an animal host. An infection caused by a micro-organism, especially a pathogen, may therefore be prevented by administering an effective dose of an attenuated bacterium according to the invention. The bacterium then expresses a heterologous protein or proteins capable of raising antibody to the micro-organism. The dosage employed will be dependent on various factors including the size and weight of the host, the type of vaccine formulated and the nature of the heterologous protein.

An attenuated bacterium according to the present invention may be prepared by transforming an attenuated bacterium with a DNA construct as hereinbefore defined. Any suitable transformation technique may be employed, such as electroporation. In this way, an attenuated bacterium capable of expressing a protein or proteins heterologous to the bacterium may be obtained. A culture of the attenuated bacterium may be grown under aerobic conditions. A sufficient amount of the bacterium is thus prepared for formulation as a vaccine, with minimal expression of the heterologous protein occurring.

The expression vector is provided with appropriate transcriptional and translational control elements including, besides the <u>htrA</u> promoter, a transcriptional termination site and translational start and stop codons and an appropriate ribosome binding site is provided. The vector typically comprises an origin of replication and, if desired, a selectable marker gene such as an antibiotic resistance gene. The vector may be, for example, a plasmid.

The invention will now be illustrated, but not limited, by reference to the following examples, and the accompanying drawings, in which:

Figure 1 is a schematic illustration of the construction of a plasmid pHTRA1 containing the <u>htrA</u> promoter in accordance with one aspect of the invention;

Figure 2 is a schematic illustration of the construction

of a plasmid pHTRA2 containing the htrA promoter and DNA encoding the tetanus toxin C-fragment linked to a hinge region;

Figure 3 illustrates the structure of the plasmid pTECH2;

Figure 4 illustrates the structure of the intermediate plasmid pBD907;

Figure 5 shows the structure of the plasmid pHTRA1 prepared in accordance with the scheme shown in Figure 1;

Figure 6 shows the structure of the product plasmid pHTRA2 prepared in accordance with the scheme shown in Figure 2;

Figures 7A to 7B illustrate the influence of temperature shifts on the promoters nirB, groE and hrtA; and

Figure 8 shows the expression of lacZ from htrA, nirB and groE in macrophages.

EXAMPLE 1

Preparation of htrA-TetC-Hinge Construct

As can be seen from Figure 1, the starting material for the preparation of a vector containing the htrA promoter and genes coding for the tetanus toxin C fragment was the plasmid pTETnir15, the structure and preparation of which is disclosed in our earlier application PCT/GB93/01617 (Publication No. WO 94/03615) and references cited therein, e.g. WO-A-92159689.

The pTETnir15 plasmid contains the nirB promoter linked

to the gene encoding the C-fragment of tetanus toxin (TetC). As shown in Figure 1, pTETnir15 was digested with SacII and BamHI and the resulting 2.9kb and 813bp fragments were gel-purified. The 2.9kb fragment was ligated with a 1.74kb fragment derived from the B. pertussis filamentous haemagglutinin (FHA) gene, the fragment having the sequence shown in SEQ.ID.NO.7. The resulting plasmid was designated pBD907 and the restriction map of the plasmid is shown in Figure 5. The purpose of preparing the intermediate plasmid pBD907 was to remove the EcoRI site present in the TetC fragment in order that the nirB promoter sequence could be replaced by the htrA promoter sequence. This was achieved by digesting plasmid pBD907 with EcoRI and BglII. The resulting 4535bp fragment was gel-purified and ligated with the following 55bp oligonucleotides containing the htrA promoter:

Oligo-1 5' AATTCTATTCCGGAACTTCGCGTTATAAAATGAATGTGACGTACACAGCAATTTA (SEQ.ID.NO.2)

Oligo-2 3' GATAAGGCCTTGAAGCGCAATATTTTACTTACACTGCATGTGTCGTTAAATCTAG (SEQ.ID.NO.3)

The presence of the promoter in the resulting intermediate plasmid pINT was confirmed by DNA sequencing. The plasmid pINT was then digested with SacII and BamHII and ligated to the 813bp fragment from pTETnir15 to form plasmid pHTRA1. The DNA sequence of pHTRA1 is shown in SEQ.ID.NO.4;

the <u>htrA</u> region which is defined by the first 55 base pairs, has the sequence AATTCTATTCCGGAACTTCGCGTTATAAAATGAATCTGACGTACACAGCAATTTA (SEQ.ID.NO.1).

In relation to SEQ.ID.NO.4, GAACTT is -35 box, and TCTGA is -10 box. At 513 and 2235 base pairs respectively are SacII and AlwN 1 restriction sites.

Plasmid pHTRA1 was used to transform <u>Salmonella typhimurium</u> strain BRD509 (deposited under accession number NCTC ....) and the resulting strain, designated BRD935, was checked for expression of TetC fragment by standard methods. Strain BRD935 has been deposited at the National Collection of Type Cultures, Colindale, United Kingdom on... under the accession number..........).

As shown in Figure 2, plasmid pHTRA1 was used to prepare a modified construct in which a "hinge" region is present at the C-terminal of the TetC fragment. The nucleotide sequence representing the "hinge" region was obtained from plasmid pTECH2 which has the DNA sequence set forth in SEQ.ID.NO.5, and possesses <u>SacII</u> and <u>AlwNI</u> restriction sites at positions 533 and 2304 respectively. The preparation of this plasmid is disclosed in our earlier Application PCT/GB93/01617 (Publication No. .....)

The pTECH2 plasmid comprises the <u>nirB</u> promoter region

linked to the tetanus toxin C fragment which, at its 3' terminal, is linked via a BamHI restriction site to a hinge region encoded a Gly-Pro-Gly-Pro repeat motif along with a number of restriction sites allowing the insertion of genes encoding further polypeptides. A 1.7kb fragment encoding the hinge region and part of the tetanus toxin C fragment region was removed from pTECH2 through digestion with SacII and AlwNI and purified. The DNA sequence of the resulting fragment is shown in SEQ.ID.NO.6.

Plasmid pHTRA1, which encodes the htrA promoter and the tetanus toxin C fragment, but includes no hinge, was digested with SacII and AlwNI and the resulting 2kb fragment was gel-purified.

The 1.7kb fragment (SEQ.ID.NO.6) from pTECH2 and the 2kb fragment from the pHTRA1 were ligated to form plasmid pHTRA2 which incorporates a htrA promoter operably linked to the gene for tetanus toxin C fragment, having at the 3' terminal thereof the hinge region.

An attenuated Salmonella typhimurium strain was transformed with vector pHTRA2 and after selection by means of standard techniques, the salmonella strain BRD1062, harbouring the plasmid pHTRA2 was isolated.

Plasmid pHTRA2 serves as an intermediate for the preparation of constructs coding for a fusion protein linked by the hinge region. Thus, in accordance with the techniques described in our earlier Application No. PCT/GB93/01617,

further proteins can be cloned into the restriction endonuclease sites on the hinge region.

MATERIALS AND METHODS

Bacterial Strains

E.coli HB101 and BRD509 (an attenuated S. typhimurium aroA aroD strain (Deposited under accession number NCTC ....) were used throughout the experiments. The bacteria were grown in Luria broth (LB) or LB solidified with 1.6% w/v agar supplemented with appropriate antibiotics.

DNA Manipulations

Plasmid DNA was purified by the alkaline lysis method (R. Maniatis, et al., 1982 Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, New York). Restricted DNA fragments were purified from agar gels by the method of Tautz and Rentz (1983, "An optimised freeze-squeeze method for the recovery of DNA Fragments from agarose gels". Analytical Biochem., 132, 14-19). Restriction enzymes were supplied by Boehringer Mannheim, Germany and New England Biolabs, USA and were used according to the manufacturer's instructions.

DNA Sequencing

DNA for double stranded sequencing was isolated by the method of Stephen et al. (1990, A Rapid Method for Isolating High Quality Plasmid DNA Suitable for DNA Sequencing, Nucleic Acid Research, 18, No. 24, p 7463). Sequencing was carried out using a Sequenase Version 2 kit (USB) and was used according to the manufacturer's instructions.

Oligonucleotides

These were synthesised on a SAM1 oligonucleotide synthesiser (Biolabs, UK).

EXAMPLE 2

Preparation of htrA-lacZ Construct

The properties of the htrA promoter were composed with two other inducible promotors, namely the nirB and groE promoters.

Sub-cloning of lacZ downstream to nirB, htrA and groE promoters

A DNA fragment encoding a promoterless lacZ gene was purified from plasmid pMAC1871 (Pharmacia) by the low melting point agarose technique, following cleavage of the plasmid with restriction enzymes SalI and BamHI [14]. Plasmids pTETnir-15 [S.N. Chatfield *et al*, Bio/Technology 10, 888-892]

and pTEThtrA-1 harbouring the nirB and htrA promoters respectively, were digested with SalI and BamHI endonucleases and the purified lacZ encoding fragment was cloned, in-frame, downstream of the promoters. Plasmid pRZ-PES was used to measure expression of β-galactosidase (β-gal) from the groE promoter. pRZ-PES contains the E. coli groE-operon promoter upstream of groES and lacZ genes. It was constructed by sub-cloning a 2.1 Kb EcoRI-HindIII fragment carrying the operon from plasmid pOF39 [O. Fayet *et al* J. Bacteriol. 171, 1379-1385 (1989)] into pUC19. A novel BglII site was then introduced between the groES and groEL genes using site directed mutagenesis. The EcoRI-BglII fragment carrying the groE promoter and groES gene was cloned into EcoRI-BamHI cut promoter-probe plasmid pRF5255 [P.F. Lambert *et al* J. Bacteriol. 162, 441-444 (1985)] to give plasmid pRZ-PES. Plasmids, prepared in S. typhimurium LB5010 (r^-m^+) [L.R. Bullas *et al* J. Bacteriol. 256, 471-474 (1983)], were introduced into S. typhimurium strain BRD915 (S. typhimurium SL1344 htrA) [S.N. Chatfield *et al* Microbial Pathog. 12, 145-151 (1992)] using electroporation. Lac positive recombinants were screened on L agar plates containing ampicillin and X-gal.

Effect of changes in environmental conditions on lacZ expression

Bacterial strains harbouring the recombinant plasmids were grown overnight in L-broth, with skaing at 30°C. The

cultures were diluted 1:50 and growth was allowed to continue for an additional 3 hours at 30°C until an OD_{600} of 2.8-3.4 was reached. 0.2 ml of each culture was stored at 4°C and used to determine the base-line of β-gal activity. The remaining portions of the cultures were then shifted to different growth conditions as described below and samples were taken at 0, 2, 4, 6 and 24 hours, unless otherwise specified. At each time point the OD_{600} was determined and the bacteria were stored at 4°C prior to performing a β-gal assay.

Measuring expression in infected HEp-2, Caco-2 and THP 1-macrophage cell lines

Cells were seeded at approximately 10^5 cell per well in twenty four well plates and grown overnight in Dolbecco's modified Eagles medium, without phenol red (ICN Flow), supplemented with 10% (vol/vol) fetal calf serum and 2 mM glutamine at 37°C, in an atmosphere of 5% CO_2. 10^8 CFU bacteria of the diluted overnight culture were added to the tissue culture cells and incubated at 30°C. At various time points samples of the tissue culture medium were taken to measure β-gal activity in the extra cellular bacteria. The numbers of bacteria in each sample were determined by viable count and the corresponding OD_{600} was determined using a standard curve. Infected cells were washed with phosphate buffer saline (PBS) and incubated for an additional hour in the presence of 200 mg/ml of gentamicin to kill extra cellular

bacteria. Thereafter, the cells were lysed using sterile distilled water and vigorous pipetting. β-gal activity was determined for each cell lysate. The numbers of bacteria in each lysate were determined by viable count and the corresponding OD_{500} values were determined using a standard curve.

RESULTS

Expression from each of the promoters selected for this study is sensitive to changes in environmental conditions. nirB has been shown previously to respond to changes in anaerobicity. Initial experiments were performed to assess the levels of lacZ expression from each of the promoters, resident on similar multicopy plasmids, harboured within Salmonella vaccine strain BRD915. The influence of temperature shifts on the different promoters is shown in Figure 7. Temperature shifts from 30°C to 37°C (Figure 7A) resulted in an increase in β-gal enzyme units when lacZ was expressed from the nirB and htrA promoters. No significant increase in β-gal units was detected from the groE promoter. A temperature shift from 30°C to 42° resulted in an increase in the number of β-gal units from all three promoters. The rate of the increase in the level of β-gal was faster from htrA and nirB compared with groE (Figure 7B). Temperature shifts from 37°C to 42°C resulted in the induction of both nirB and htrA promoters, with more moderate increase in β-gal

units from groE promoter (Figure 7C).

Expression of β-gal from the different promoters was also tested by selecting for bacteria that had entered eukaryotic cells. HEp-2, Caco-2 and THP-1 macrophage cell lines were infected with 10^8 bacteria and incubated at 30°C. The number of β-gal units, determined three hours after infection of HEp-2, showed that expression of lacZ from both htrA and nirB promoters was significantly enhanced (Figure 2). However there was no detectable increase in lacZ expression from groE promoter. Similar results were obtained in infected Caco-2 cells (not shown). In contrast, in the macrophage's intracellular environment, all three promoters were induced (Figure 8). nirB promoter was most affected and groE promoter was least affected (Figure 8). When the number of β-gal units in the extra-cellular medium of either cell line was determined, no increase in the enzyme activity was seen (not shown).

Since growth within macrophages was found to influence expression from all three promoters, their sensitivity to hydrogen peroxide, commonly found within the phagosome of macrophages, was monitored. Incubating the bacteria at 30°C in 100 μM hydrogen peroxide resulted in no significant effect on the groE and nirB promoters. In contrast, the level of β-gal was increased from the htrA promoter reaching 10 U above base-line level by 4 hours. This was followed by a rapid decrease to base-line levels by 6 hours (not shown).

Constitutive expression of lacZ from plasmid pLK [M. Szabo *et al* J. Bacteriol. 174, 7245-7252] was not significantly affected by any of the environmental conditions (not shown).

In this study three environmentally regulated promoters were used to express lacZ gene under different growth conditions. The promoters are representatives of three classes of inducible bacterial promoters: the anaerobically inducible E. coli nirB, the σ^E dependent htrA and σ^{32}-dependent groE. Expression from the nirB promoter is dependent on the transcription factor FNR which binds between positions -52 and -30 upstream from transcription start. In some cases FNR dependent transcription is modulated together with a second transcription factor NarL. However, plasmid pTETnir-15 used here contains only the FNR dependent bind site.

Bacterial respond to environmental stress conditions by rapid change in the rate of synthesis of many proteins. In many cases the transit induction rapidly adjusts the protein levels to a new steady state. In this study we tested the influence of environmental conditions on the level of β-gal. We found that temperature shift had a greater effect on htrA promoters compared with groE. This result is in line with the fact that in vivo the htrA promoter is induced before groE (which is σ^{32}-dependent) and together with σ^{32}, by σ^E containing RNA polymerase [11, 12]. Surprisingly, the nirB promoter was also enhanced by elevated temperature. Although it is possible that at high temperature the concentration of oxygen

in the growing media is reduced the fact that the temperature shift from 30°C to 37°C brings rapid increase in the β-gal units expressed from the nirB promoter may suggest that FNR, like other stress protein modulators, responds to a number of environmental stimuli. Similarly, htrA was also induced under anaerobic growth conditions, and therefore it seems that this promoter is either being regulated by factors other than σ^E, or that σ^E is being activated also at low oxygen tension.

For S. typhimurium to retain virulence the bacteria has to be able to survive in macrophages. This survival is dependent on the ability of the bacteria to tolerate a range of toxic killing machanisms including the production of hydrogen peroxide. Unlike E. coli htrA mutants, S. typhimurium htrA mutants have been found previously not to be killed by elevated temperature, but rather to have impaired ability to survive significant levels of hydrogen peroxide. Interestingly, the htraA promoter was the only one of the test promoters whose expression was increased in the presence of hydrogen peroxide.

In order to determine the influence of the intracellular environment, the level of expression from the three different promoters was monitored after Salmonella harbouring the test plasmids had entered a number of different cultured eukaryotic cell lines. Bacteria were grown in vitro and used to infect eukaryotic cells at 30°C since a temperature shift from 30°C to 37°C dramatically induced both the htrA and the nirB

promoters. We found that while the level of β-gal expression from both the <u>nirB</u> and <u>htrA</u> promoters increased in all the cell lines tested, <u>groE</u> promoter was induced only in infected macrophages.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: MEDEVA HOLDINGS BV

(B) STREET: CHURCHILL-LAAN 223

(C) CITY: AMSTERDAM

(E) COUNTRY: THE NETHERLANDS

(F) POSTAL CODE (ZIP): 1078 ED

(ii) TITLE OF INVENTION: VACCINES

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: GB 9401795.1

(B) FILING DATE: 31-JAN-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 55 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Salmonella typhimurium

(ix) FEATURE:

(A) NAME/KEY: promoter

(B) LOCATION: 1..55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 55 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AATTCTATTC CGGAACTTCG CGTTATAAAA TGAATGTGAC GTACACAGCA ATTTA 55

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 55 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GATAAGGCCT TGAAGCGCAA TATTTTACTT ACACTGCATG TGTCGTTAAA TCTAG 55

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3712 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: htrA promoter

(B) LOCATION: 1..55

(ix) FEATURE:

(A) NAME/KEY: SacII restriction site

(B) LOCATION: 513

(ix) FEATURE:

(A) NAME/KEY: AlwN 1 restriction site

(B) LOCATION: 2235

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3769 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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CAACAACGAT ATTATCTCCG ACATCTCTGG TTTCAACTCC TCTGTTATCA CATATCCAGA 240

TGCTCAATTG GTGCCGGGCA TCAACGGCAA AGCTATCCAC CTGGTTAACA ACGAATCTTC 300

TGAAGTTATC GTGCACAAGG CCATGGACAT CGAATACAAC GACATGTTCA ACAACTTCAC 360

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ATTAACCTAT AAAAATAGGC GTATCACGAG GCCCTTTCGT CTTCAAGAA 3769

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1766 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

(A) NAME/KEY: hinge region

(B) LOCATION: 923..934

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1736 base pairs

(B) TYPE: nucleic acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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CGACCTGAGC GCAGCGCGCG GCGCGGATAT CTCCGGCGAG GGGCGCGTCA ATATCGGCCG 960

TGCGCGCAGC GATAGCGATG TGAAGGTCTC CGGGCACGGC GCCTTGTCGA TCGATAGCAT 1020

GACGGCCCTC GGTGCGATCG GCGTCCAGGC AGGCGGCAGC GTGTCGGCCA AGGATATGCG 1080

CAGCCGTGGC GCCGTCACCG TCAGCGGCGG CGGCGCCGTC AACCTGGGCG ATGTCCAGTC 1140

GGATGGGCAG GTCCGCGCCA CCAGCGCGGG CGCCATGACG GTGCGAGACG TCGCGGCTGC 1200

CGCCGACCTT GCGCTGCAGG CGGGCGACGC GTTGCAGGCC GGGTTCCTGA AATCGGCCGG 1260

TGCCATGACC GTGAACGGCC GCGATGCCGT GCGACTGGAT GGCGCGCACG CGGGCGGGCA 1320

ATTGCGGGTT TCCAGCGACG GGCAGGCTGC GTTGGGCAGT CTCGCGGCCA AGGGCGAGCT 1380

GACGGTATCG GCCGCGCGCG CGGCGACCGT GGCCGAGTTG AAGTCGCTGG ACAACATCTC 1440

CGTGACGGGC GGCGAACGCG TGTCGGTTCA GAGCGTCAAC AGCGCGTCCA GGGTCGCCAT 1500

TTCGGCGCAC GGCGCGCTGG ATGTAGGCAA GGTTTCCGCC AAGAGCGGTA TCGGGCTCGA 1560

AGGCTGGGGC GCGGTCGGAG CGGACTCCCT CGGTTCCGAC GGCGCGATCA GCGTGTCCGG 1620

GCGCGATGCG GTCAGGGTCG ATCAAGCCCG CAGTCTTGCC GACATTTCGC TGGGGGCGGA 1680

AGGCGGCGCC ACGCTGGGCG CGGTGGAGGC CGCCGGTTCG ATCGACGTGC GCGGCG 1736

CLAIMS

1. A DNA construct comprising the htrA promoter sequence operably linked to a DNA sequence encoding one or more heterologous proteins.

2. A DNA construct according to Claim 1 wherein the htrA promoter sequence is operably linked to a DNA sequence encoding a fusion protein of two or more proteins.

3. A DNA construct according to Claim 2 wherein the proteins making up the fusion are linked by means of a flexible hinge region.

4. A DNA construct according to Claim 3 wherein the htrA promoter is operably linked to a DNA sequence encoding first and second heterologous proteins wherein the first heterologous protein is an antigenic sequence comprising tetanus toxin fragment C or one or more epitopes thereof.

5. A replicable expression vector, e.g. suitable for use in bacteria, containing a DNA construct as defined in any one of the preceding Claims.

6. A process for the preparation of an attenuated bacterium which comprises transforming an attenuated bacterium with a replicable expression vector as defined in Claim 1.

7. A host cell containing in either chromosomal or extra-chromosomal form, a DNA construct as defined in any on of Claims 1 to 4.

8. A host cell according to Claim 7 which is an attenuated bacterium.

9. A vaccine composition comprising an attenuated bacterium as defined in Claim 8, or a fusion protein expressed from a construct as defined in any one of Claims 1 to 4, and a pharmaceutically acceptable carrier.

10. A method of treatment or prophylaxis of infection in a mammal, e.g. a human, which method comprises administering to the mammal an effective amount of a vaccine composition as defined in Claim 9.

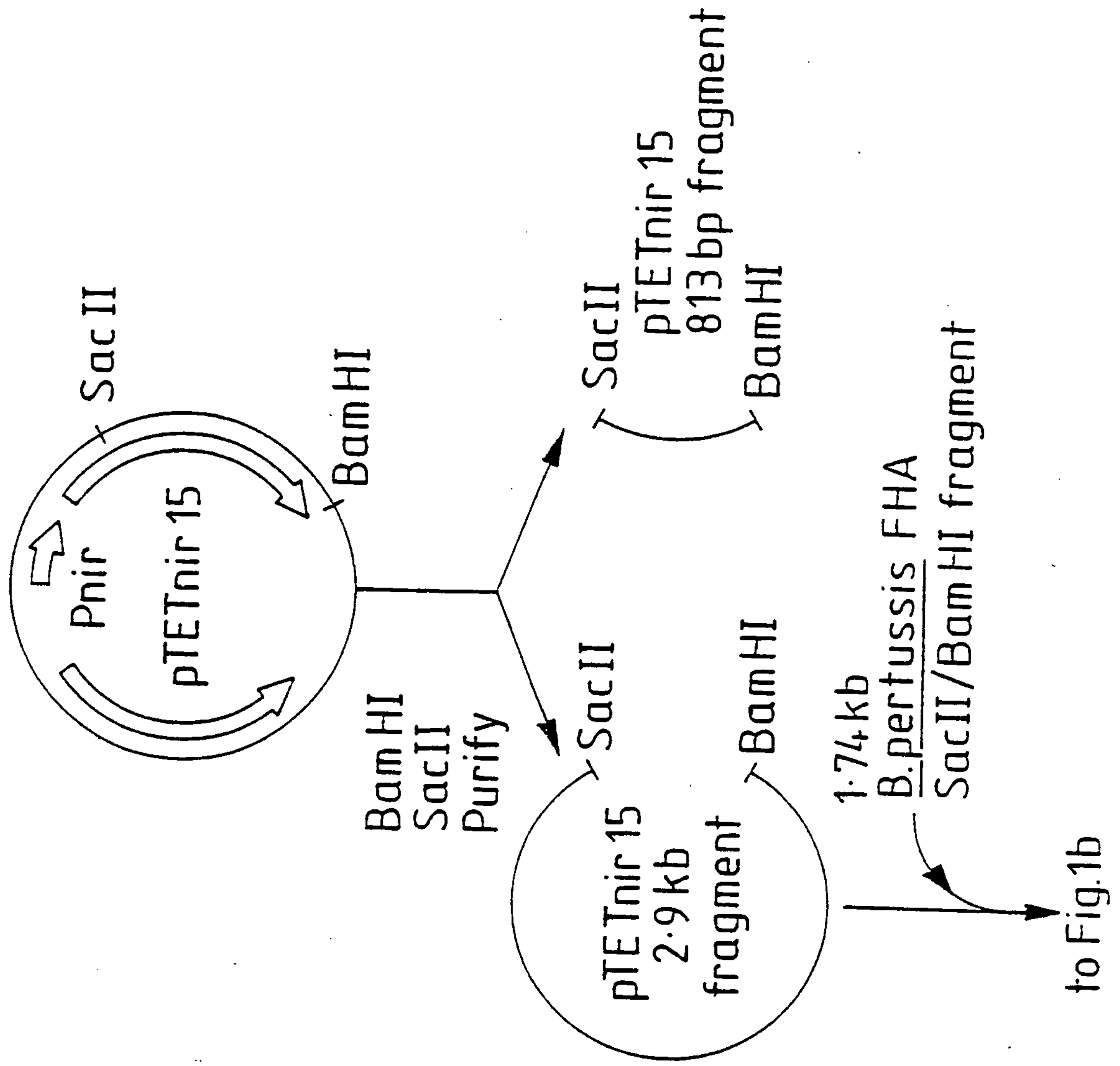

SacII
BamHI
Pnir
pTETnir 15
BamHI
SacII
Purify
SacII
pTETnir 15
813 bp fragment
BamHI
SacII
BamHI
pTETnir 15
2·9 kb
fragment
1·74 kb
B.pertussis FHA
SacII/BamHI fragment
to Fig.1b

FIGURE 1a

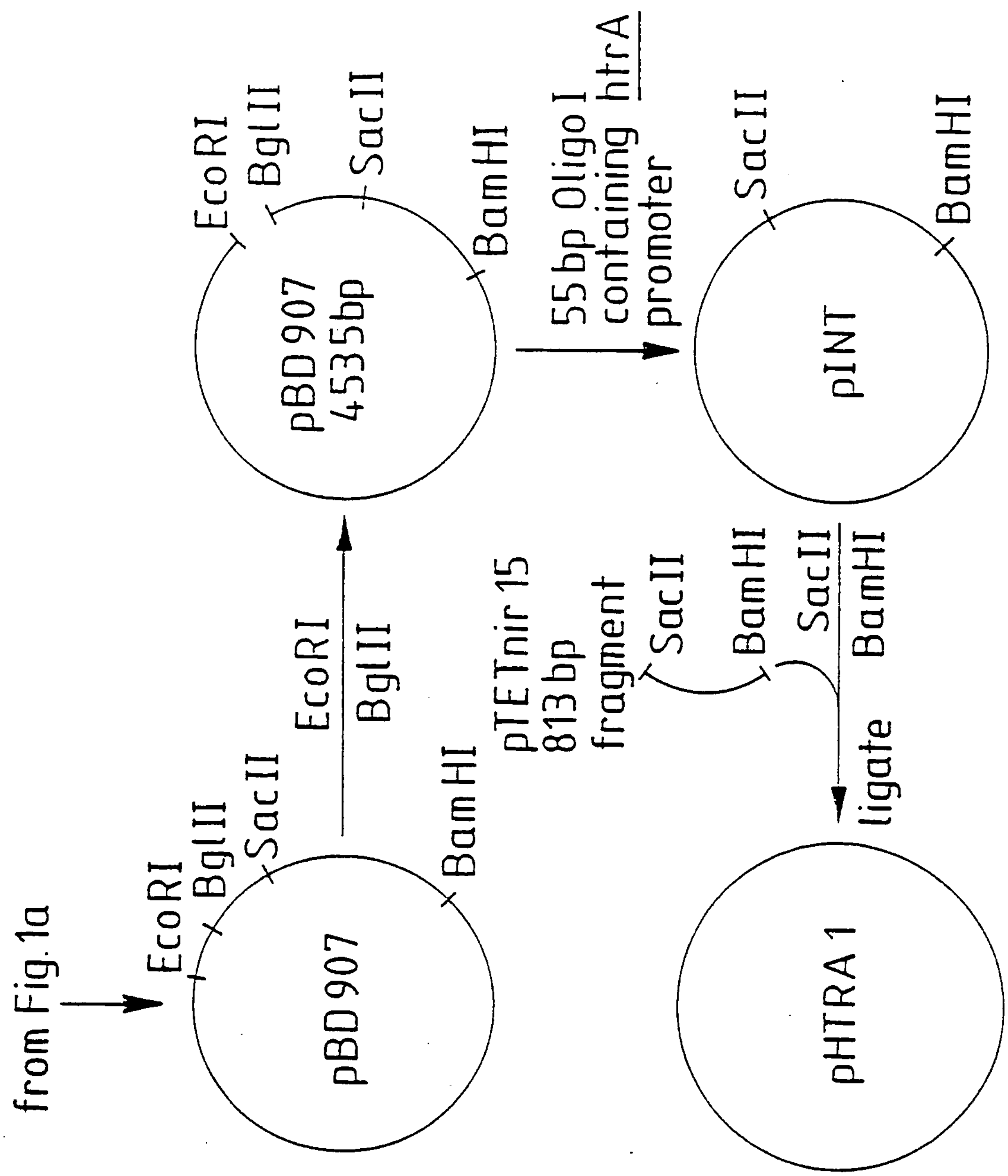
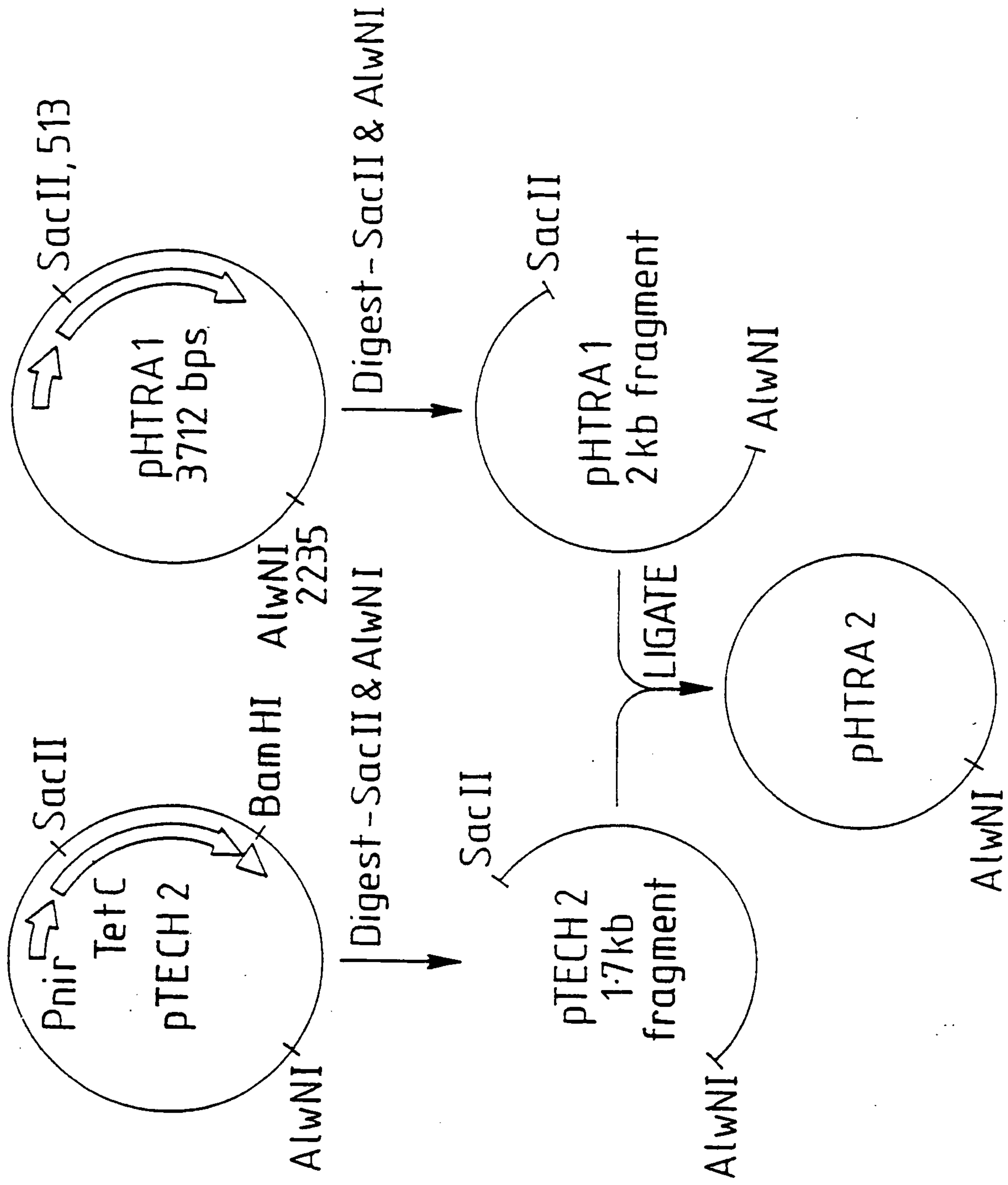

from Fig. 1a
pBD907
EcoRI
BglII
SacII
BamHI
EcoRI
BglII
pBD907
4535bp
EcoRI
BglII
SacII
BamHI
55bp Oligo I containing htrA promoter
pINT
SacII
BamHI
pTETnir15
813bp
fragment
SacII
BamHI
SacII
BamHI
ligate
pHTRA1

FIGURE 1b



Pnir
Tet C
pTECH 2
SacII
BamHI
AlwNI
Digest - SacII & AlwNI
SacII
pTECH 2
1·7kb
fragment
AlwNI
SacII, 513
pHTRA1
3712 bps
AlwNI
2235
Digest - SacII & AlwNI
SacII
pHTRA1
2 kb fragment
AlwNI
LIGATE
pHTRA 2
AlwNI

FIGURE 2

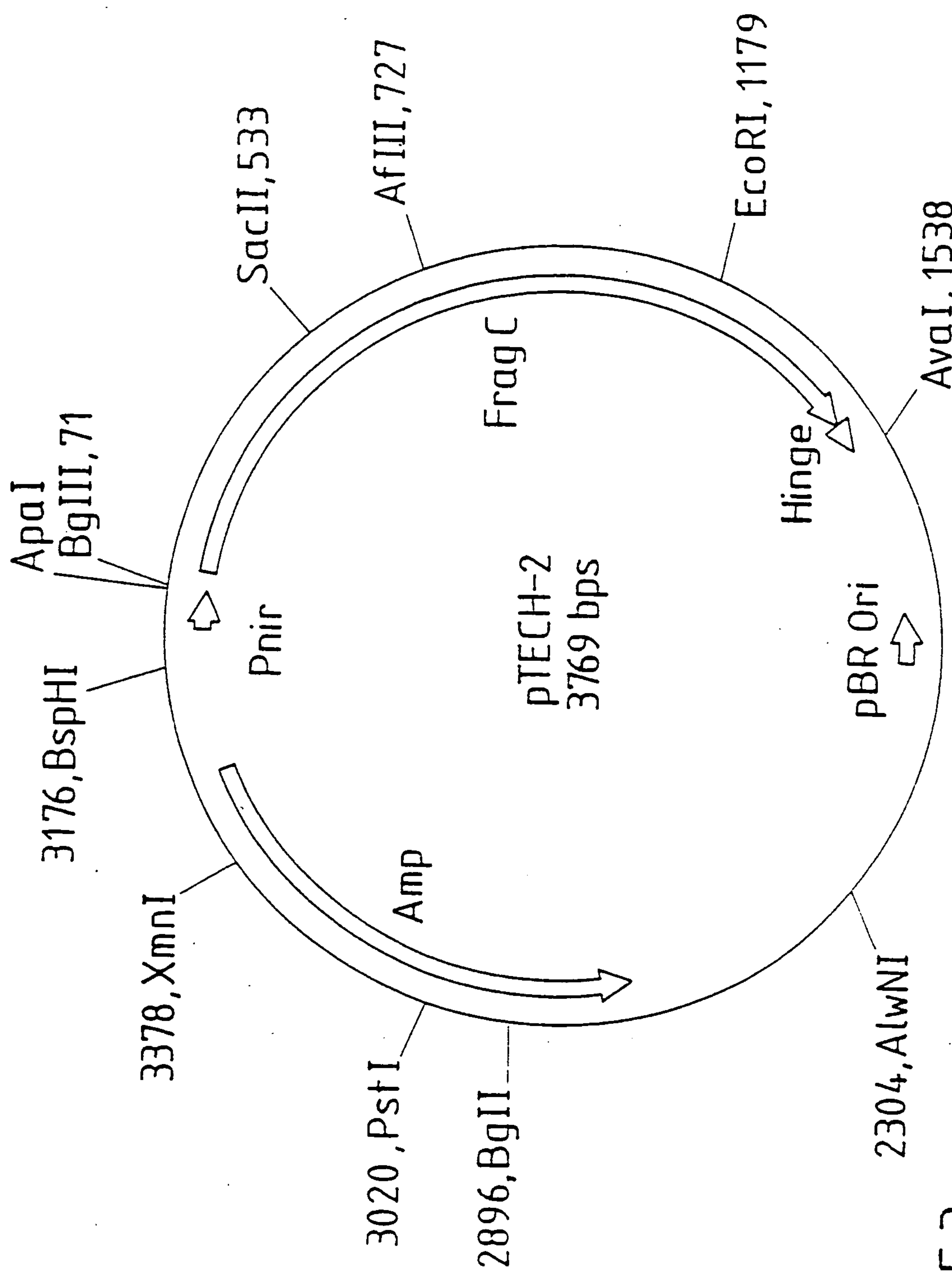

ApaI
BglII,71
SacII,533
AflII,727
EcoRI,1179
AvaI,1538
Frag C
Hinge
pBR Ori
pTECH-2
3769 bps
Pnir
3176,BspHI
3378,XmnI
Amp
3020,PstI
2896,BglI
2304,AlwNI

FIGURE 3

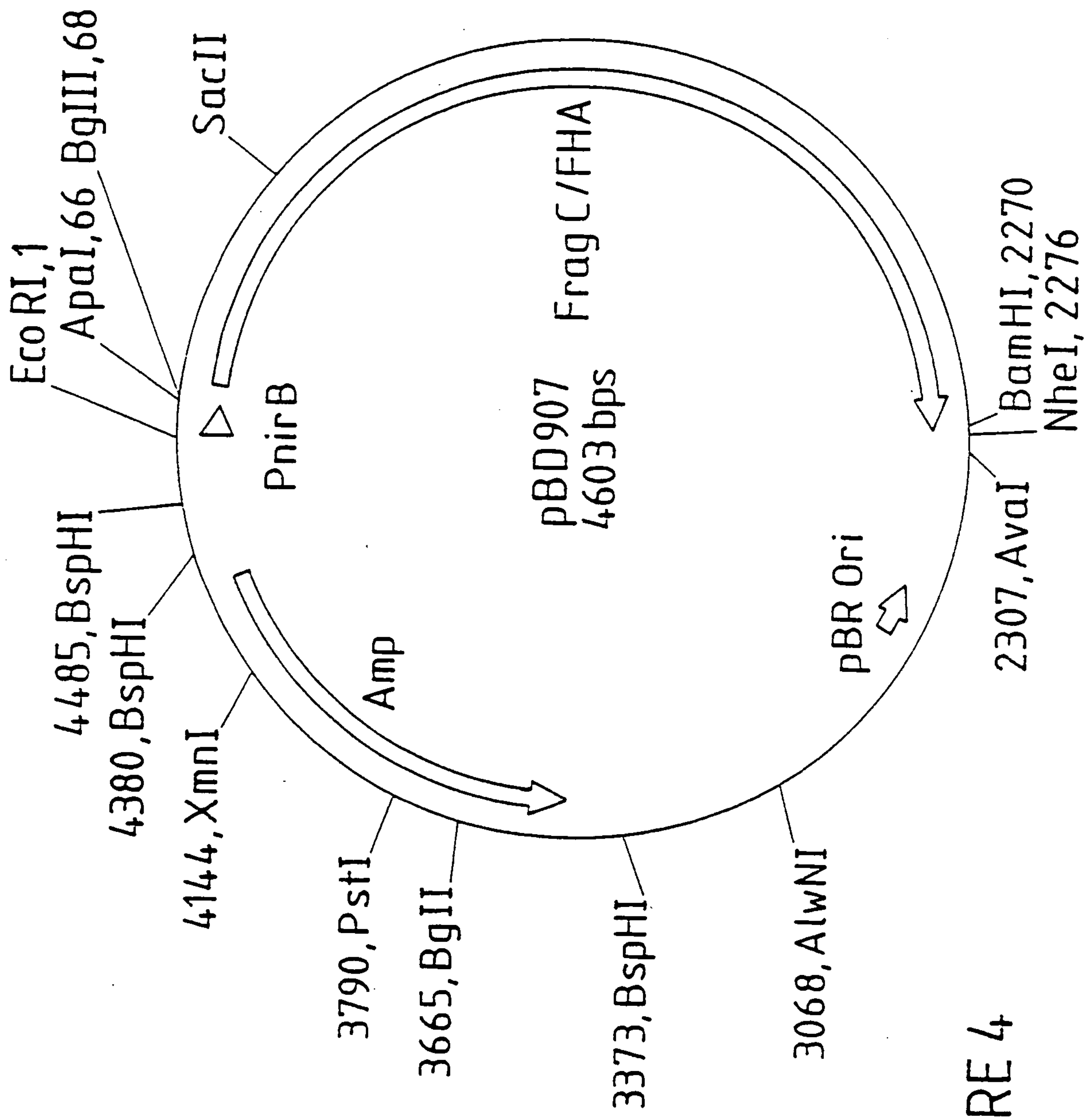

EcoRI,1
ApaI,66
BglII,68
SacII
Frag C/FHA
BamHI,2270
NheI,2276
2307,AvaI
PnirB
pBD907
4603 bps
pBR Ori
Amp
4485,BspHI
4380,BspHI
4144,XmnI
3790,PstI
3665,BglI
3373,BspHI
3068,AlwNI

FIGURE 4

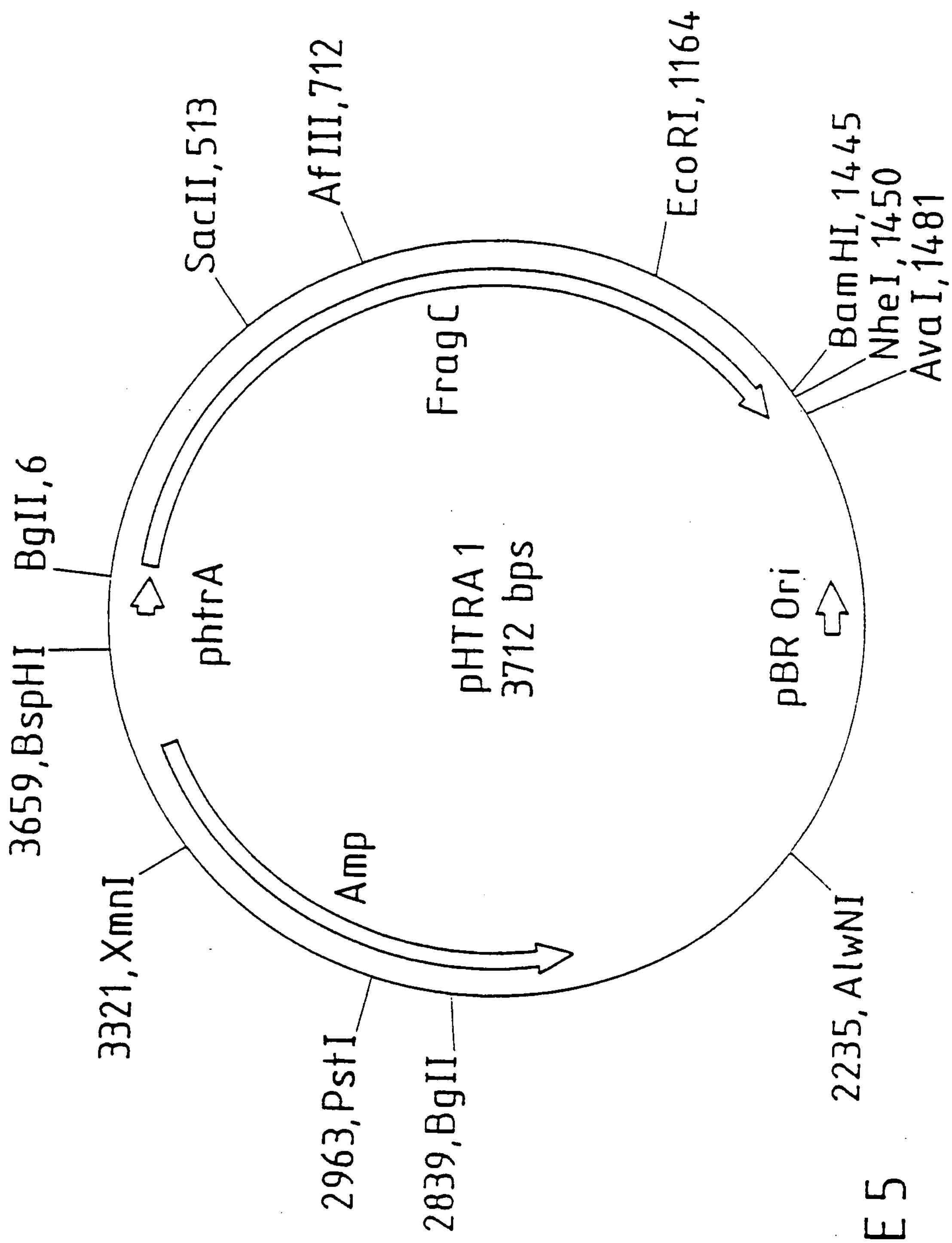

SacII,513
AflIII,712
EcoRI,1164
BamHI,1445
NheI,1450
AvaI,1481
FragC
BglI,6
phtrA
pHTRA1
3712 bps
pBR Ori
3659,BspHI
Amp
3321,XmnI
2963,PstI
2839,BglI
2235,AlwNI

FIGURE 5

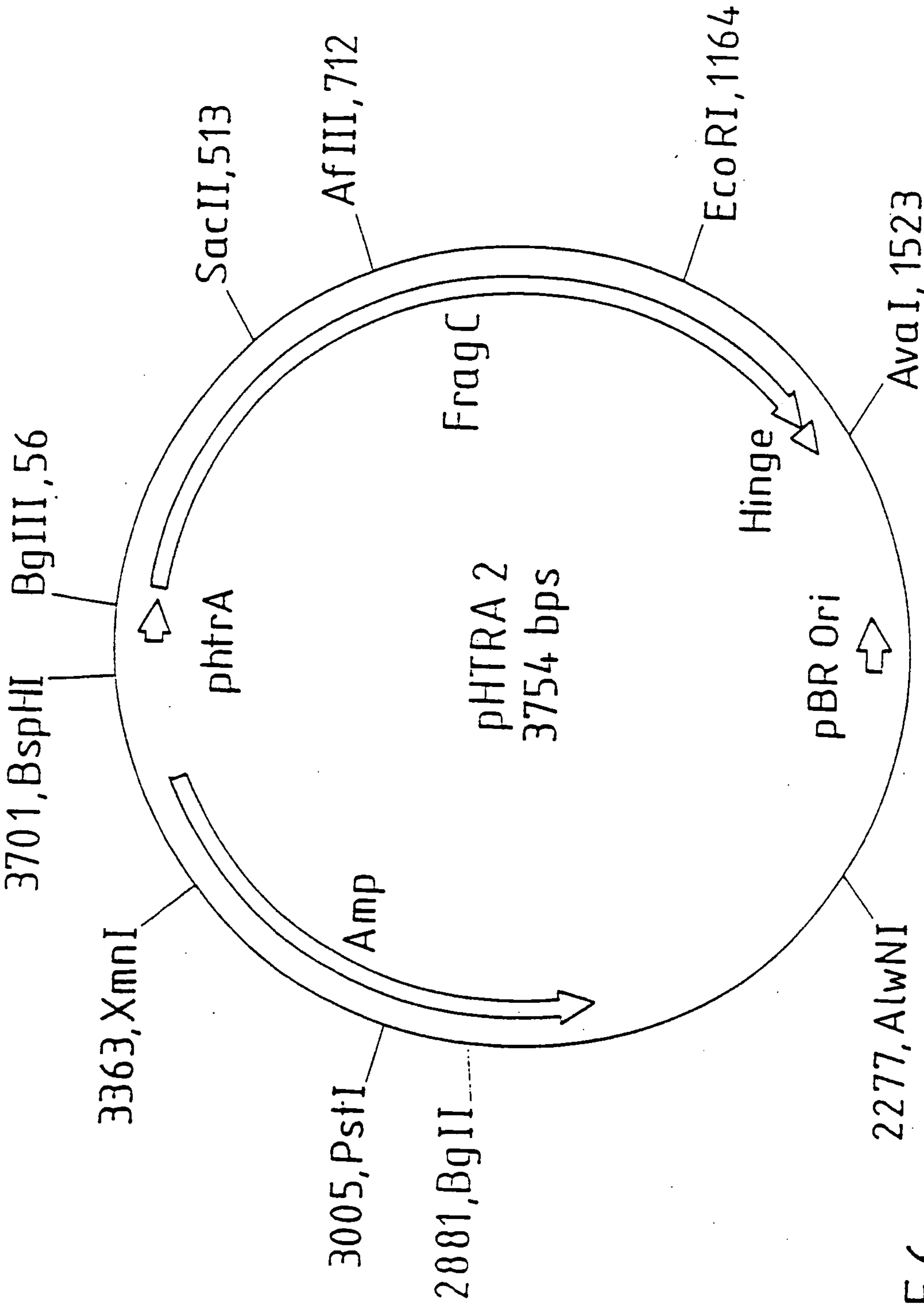

SacII,513
AflII,712
EcoRI,1164
AvaI,1523
FragC
Hinge
pBR Ori
2277,AlwNI
2881,BglI
3005,PstI
3363,XmnI
Amp
3701,BspHI
BglII,56
phtrA
pHTRA 2
3754 bps

FIGURE 6

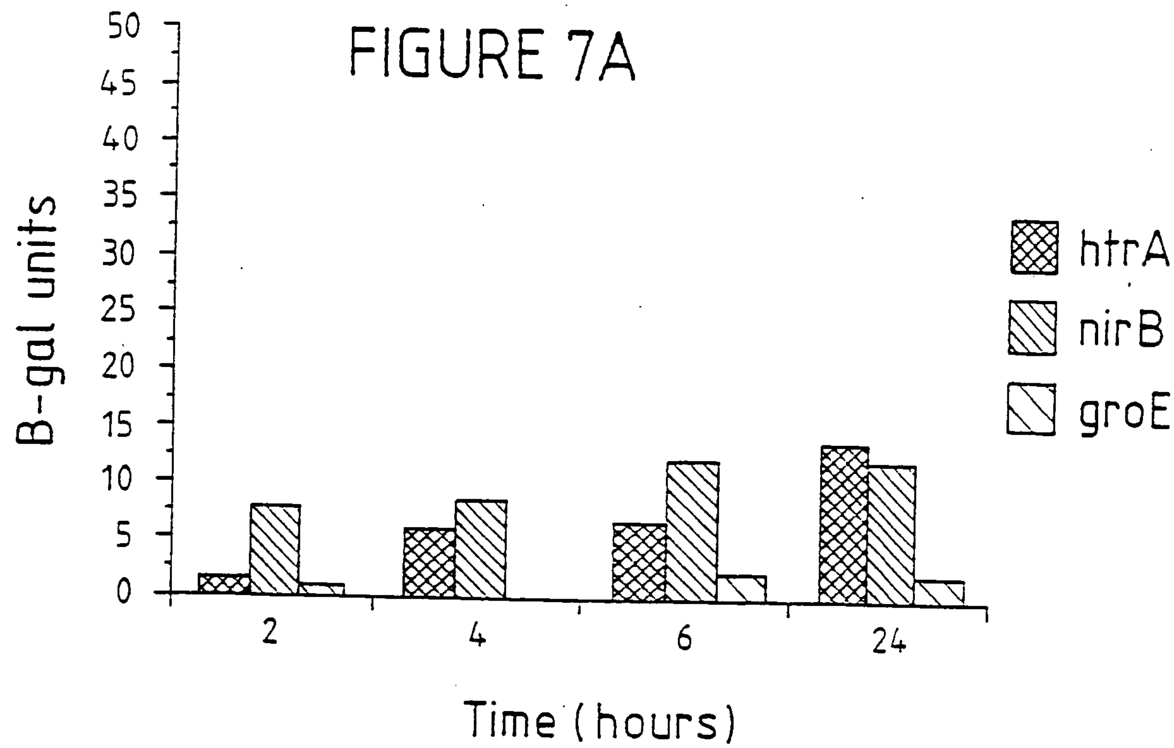


FIGURE 7A
50
45
40
35
30
25
20
15
10
5
0
B-gal units
htrA
nirB
groE
2
4
6
24
Time (hours)

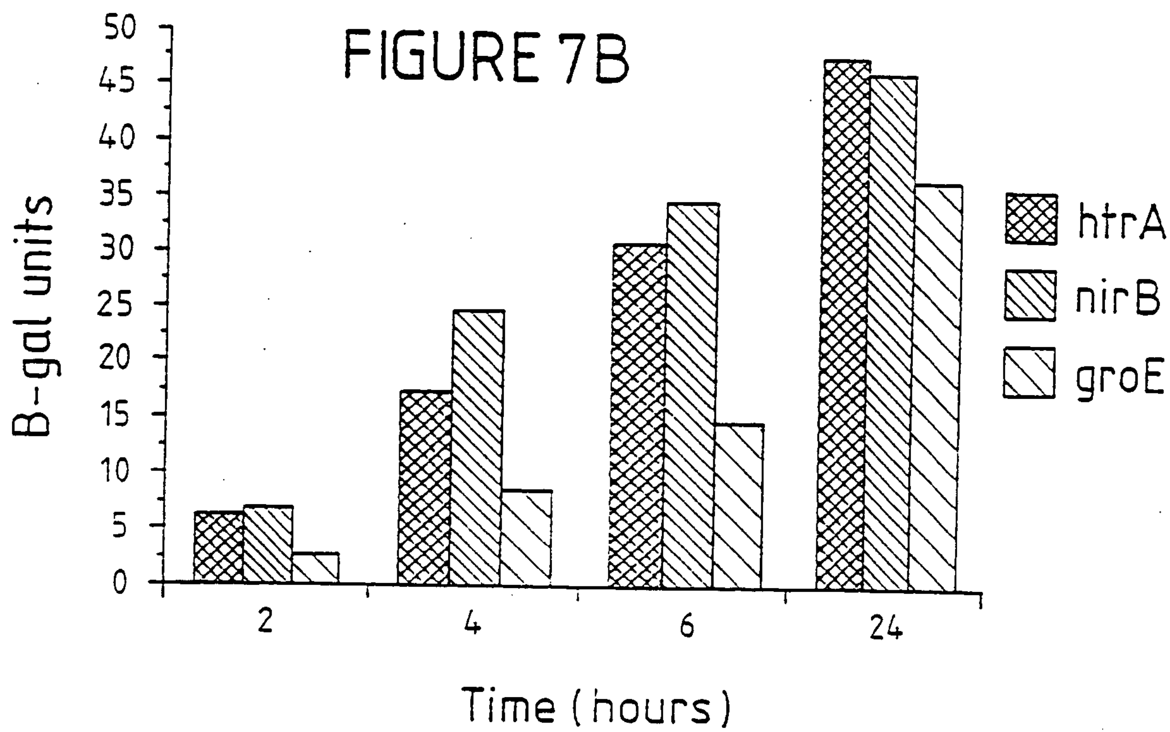
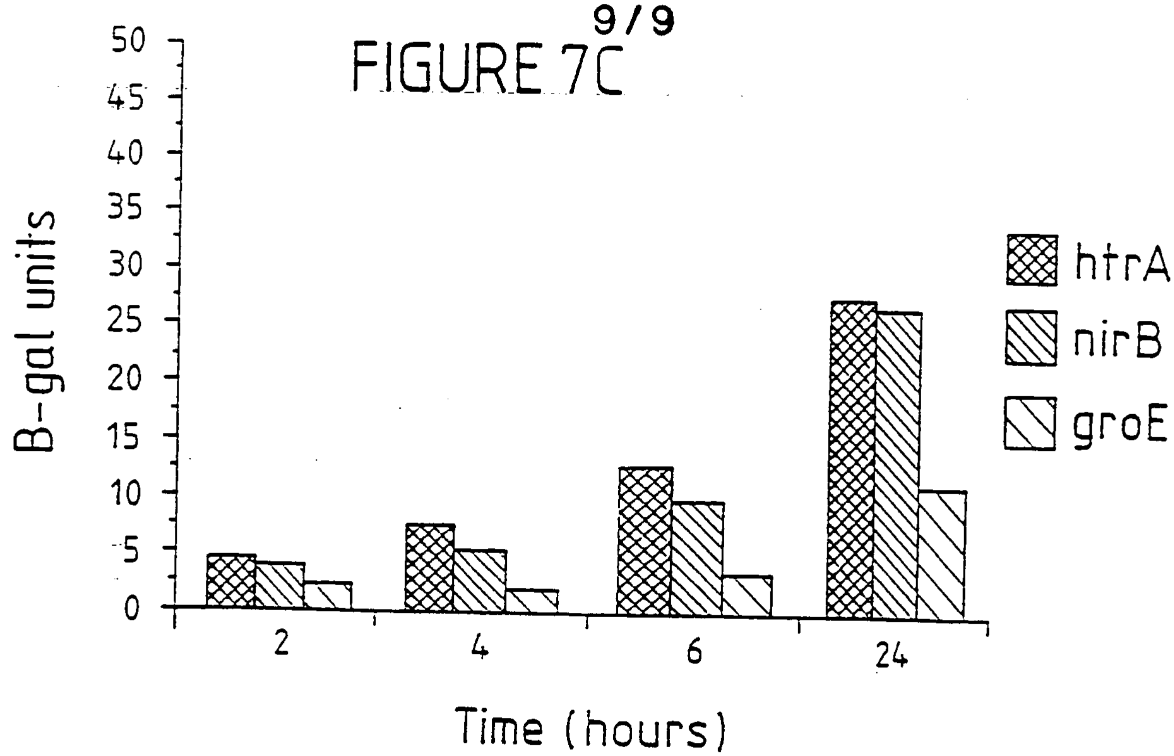


FIGURE 7B
50
45
40
35
30
25
20
15
10
5
0
B-gal units
htrA
nirB
groE
2
4
6
24
Time (hours)

FIGURE 7C



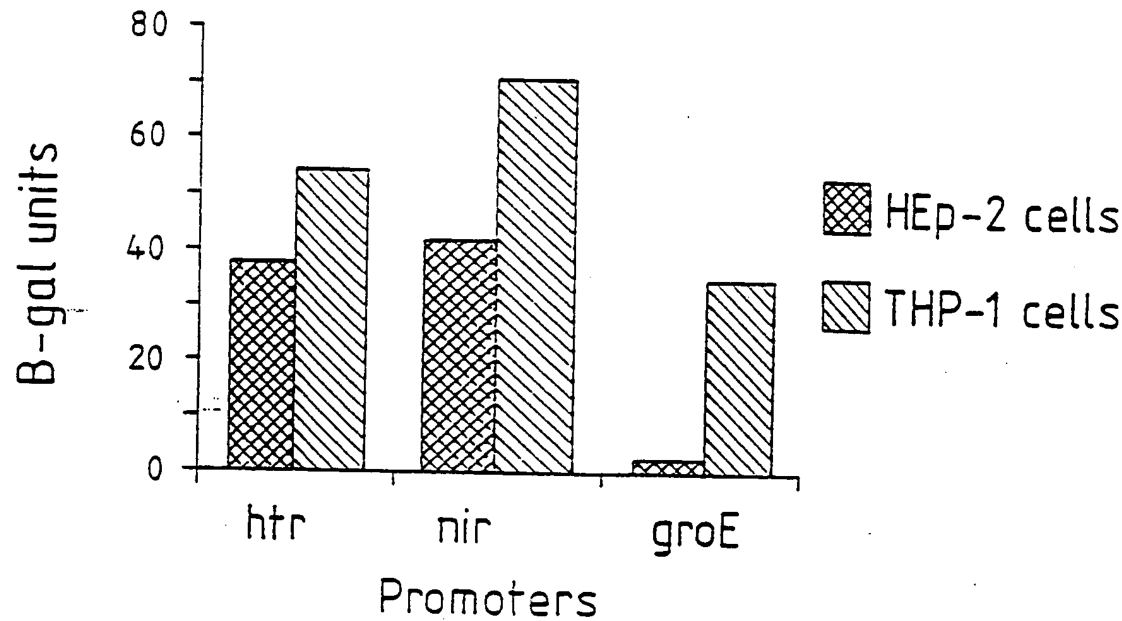


FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/00196

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C12N15/70 C12N15/74 C12N1/21 A61K39/08
//(C12N1/21,C12R1:19,C12R1:42)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH, vol. 16,no. 21, 11 November 1988 IRL PRESS LIMITED,OXFORD,ENGLAND, pages 10053-10067, B. LIPINSKA ET AL. 'Sequence analysis and regulation of the htrA gene of Escherichia coli: a sigma32-independent mechanism of heat-inducible transcription' see page 10057, line 16 - page 10066, line 25 see page 889, left column, line 5 - line 9 --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
5 April 1995	07.04.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

Inter .nal Application No
PCT/GB 95/00196

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GENES & DEVELOPMENT, vol. 3,no. 9, September 1989 CSH LABORATORY PRESS, NEW YORK,US, pages 1462-1471, J.W. ERICKSON AND C.A. GROSS 'Identification od sigmaE subunit of Escherichia coli RNA polymerase: a second alternative sigma factor involved in high-temperature gene expression' see page 1466, right column, line 4 - page 1469, right column, line 27; figure 8 ---	1-10
Y	BIOTECHNOLOGY, vol. 10,no. 8, August 1992 NATURE PUBL. CO.,NEW YORK, US, pages 888-892, S.N. CHATFIELD ET AL. 'Use of the nirB promoter to direct the stable expression of heterologous antigens in Salmonella oral vaccine strains: Development of a single-dose oral tetanus vaccine' cited in the application see page 888, right column, line 45 - line 48 see page 889, left column, line 5 - line 9 see page 891, left column, line 34 - line 39 ---	1-10
Y	NATURE, vol. 351,no. 6326, 6 June 1991 MACMILLAN JOURNALS LTD., LONDON,UK, pages 456-460, C.K. STOVER ET AL. 'New use of BCG for recombinant vaccines' see page 458, right column, line 44 - page 459, left column, line 35 see page 457, right column, line 1 - line 15 ---	1-10
Y	WO,A,92 15689 (WELLCOME FOUND) 17 September 1992 see page 2, line 1 - line 10 see page 5, line 6 - page 11, line 3 ---	1-10
Y	WO,A,89 06974 (PRAXIS BIOLOG INC) 10 August 1989 see page 76, line 1 - page 81, line 4; claims 1-107; tables 12-15 see page 26, line 25 - page 27, line 14 see page 25, line 12 - page 26, line 9 ---	1-10
1 9 Y	EP,A,0 432 965 (SMITHKLINE BEECHAM CORP ;US OF AMERICA AS RESPRESENTED (US); BIOME) 19 June 1991 see page 16, line 24 - line 29 --- -/--	1-10

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,91 15572 (WELLCOME FOUND) 17 October 1991 the whole document ---	1-10
P,Y	WO,A,94 03615 (MEDEVA HOLDINGS B V ;KHAN MOHAMMED ANJAM (GB); HORMAECHE CARLOS ES) 17 February 1994 cited in the application see page 2, line 15 - line 17 see page 4, line 9 - line 15 ---	1-10
P,X	BIOLOGICAL ABSTRACTS, vol. 99, no. 007, Philadelphia, PA, US; abstract no. 095219, see abstract & FEMS MICROBIOL. LETTERS, vol. 126,no. 1, 1995 pages 97-102, P. EVEREST ET AL. 'Expression of LacZ from the htrA, nirB and groE promoters in Salmonella vaccine strain: influence of growth in mammalian' -----	1-10

1

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 10 (as far as in vivo methods are concerned) is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9215689	17-09-92	AU-A-	1350892	06-10-92
		CZ-A-	9301005	19-01-94
		EP-A-	0574466	22-12-93
		WO-A-	9215688	17-09-92
		HU-A-	66833	30-01-95
		JP-T-	6505158	16-06-94
WO-A-8906974	10-08-89	AT-T-	109008	15-08-94
		AU-B-	634153	18-02-93
		AU-A-	3065489	25-08-89
		DE-D-	68917126	01-09-94
		DE-T-	68917126	02-02-95
		EP-A-	0399001	28-11-90
		JP-T-	3502691	20-06-91
EP-A-0432965	19-06-91	AU-B-	634837	04-03-93
		AU-A-	6777490	13-06-91
		CA-A-	2031468	09-06-91
		CN-A-	1053814	14-08-91
		JP-A-	6073097	15-03-94
WO-A-9115572	17-10-91	AU-A-	7541791	30-10-91
		CA-A-	2079463	01-10-91
		EP-A-	0524205	27-01-93
		HU-A-	65496	28-06-94
		NZ-A-	237616	25-03-94
WO-A-9403615	17-02-94	AU-B-	4719393	03-03-94
		FI-A-	950396	30-01-95